Cloning by Ophiuroid Echinoderm Larvae

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Abstract. Larvae of the brittle star *Ophiopholis aculeata*, common to the North Pacific coast of the United States, and an unidentified species of ophiuroid, collected from waters off the eastern coast of Florida, undergo asexual reproduction of the primary larva to produce a secondary larval clone. Generation of a secondary larva begins with the release of the larval posterolateral arms, which are initially retained by the settled juvenile. In *O. aculeata*, the released arms regenerate all the structures typical of the primary ophiopluteus. Tissue and energy reserves required for formation of the secondary feeding larva appear to be supplied by the absorption and reorganization of part of the posterolateral arms. Various developmental stages of the unidentified ophiopluteus were collected from plankton samples taken off the coast of Florida. These included just-released posterolateral arms, plutei, and metamorphosed juveniles with the posterolateral arms still attached. The identification of regenerating arms from the plankton demonstrates that asexual reproduction by ophiuroid larvae is not restricted to a single, laboratory-cultured species. In both *O. aculeata* and the unidentified Atlantic ophiopluteus, cloning involves the dedifferentiation of primary larval tissue and a developmental progression similar to that followed by the zygote, although it is not known whether the formation of the secondary larva follows the same pathway utilized by the primary larva or a novel developmental program. Asexually produced secondary larvae of *O. aculeata* undergo metamorphosis, settle to the benthos, and initiate a tertiary larval generation, indicating that cloned larvae could be added to the population as long as environmental conditions could support a planktonic existence. This phenomenon represents an unusual potential to increase the geographic range and the number of juveniles of a given parentage in future generations without additional reproductive input from the adult.

Introduction

In 1921, Mortensen reported the appearance of unusual brittle star larvae in plankton tows taken from the waters surrounding the islands of the West Indies. He speculated that these larvae were asexual clones originating from the released posterolateral arms of settled juveniles. MacBride (1921), renowned for his contributions to echinoderm embryology, strongly rejected this interpretation, and Mortensen's observation remained unconfirmed for the next 75 years (Mladenov and Burke, 1994).

Larval cloning is a rare event in the life history of nonparasitic planktonic invertebrate larvae, but is reported for a number of species of asteroid echinoderms (Bosch *et al.*, 1989; Rao *et al.*, 1993; Jaeckle, 1994). In asteroid larvae, the formation of the asexually produced secondary propagule results from the differentiation and release of some part of the primary larval body. The propagule develops into a secondary larva that is morphologically identical to the primary larva. It is not known if the secondary larvae of asteroids are capable of settlement and metamorphosis or if continued cycles of asexual reproduction are possible.

The work presented here confirms Mortensen's (1921) earlier observations that ophiuroid larvae undergo asexual reproduction to produce secondary clones. Like asteroids, secondary larvae of *Ophiopholis aculeata* and of an unidentified ophiopluteus collected from the Western Atlantic Ocean off the coast of Florida originate from the release and development of primary larval structures, specifically, the posterolateral arms. The released arms undergo gastrulation and development similar to that of the primary embryo, but development of secondary larvae follows from the reorganization of larval tissues instead of from embryonic cells. This raises significant questions

concerning the flexibility of developmental pathways and the initiation and control of morphogenesis.

In *O. aculeata*, asexually produced secondary larvae undergo metamorphosis, settle to the benthos, and initiate a tertiary larval generation. Asexual reproduction of larval propagules has potential evolutionary and ecological ramifications. For each juvenile that survives to settlement, an additional larva is produced that not only increases the number of individuals of a given genetic lineage, but also enhances the dispersal potential of the species.

Methods and Materials

Adult specimens of *Ophiopholis aculeata* were collected during the summer of 1996 from the intertidal and subtidal zones of various sites along the shores of San Juan Island, Washington. The animals were transported to the Friday Harbor Laboratories, Friday Harbor, Washington, where spawning was induced by alternating light and dark exposure. Fertilization and culturing of the embryos and larvae followed methods outlined by Strathmann (1987). Feeding ophioplutei were kept in 2 l of 5-μm-filtered seawater in glass jars at 10C°, stirred gently by paddles, and fed cultured cells of the alga *Rhodomonas* sp. Juveniles suspended between the posterolateral arms and free-swimming released arms were removed from the jars and maintained in culture dishes. Clean filtered seawater and algae were provided every other day, and the cultures were stirred gently with a pipette several times a day to suspend food and larvae in the water column. A Wild M-5 dissecting scope and a Nikon Optiphot-2 compound microscope were used to take photographs on T-Max 100 (Kodak) film.

During May and June of 1997, plankton was collected approximately 1 mile off the coast of Ft. Pierce, Florida, by towing a 202-μm-mesh plankton net at a depth of less than 10 m. The samples were transported to the Smithsonian Marine Station in Ft. Pierce and examined for ophioplutei. In some samples, an unidentified ophiopluteus was the most abundant organism present. Plutei, juveniles with attached posterolateral arms, and free-swimming posterolateral arms in various stages of development were collected and maintained in culture dishes in filtered seawater. Larvae were fed cells of *Rhodomonas* sp. Larvae and regenerating arms were photographed with a Zeiss Photomicroscope II and T-Max 100 film.

Results

Larvae of Ophiopholis aculeata

The development of primary larvae of *O. aculeata* follows that described for other planktotrophic ophioplutei (Hendler, 1991). The onset of metamorphosis is indicated

by the appearance of the five hydrocoelic lobes of the presumptive primary podia. Metamorphosis continues while the larva remains in the water column, eventually resulting in a juvenile suspended between the two outer posterolateral arms (Fig. 1A). The juvenile retains the posterolateral arms for some undetermined period after settlement. During this time the transverse rods appear to shorten and contact is made between the proximal tip of each posterolateral arm. The posterolateral arms are released from the settled juvenile (Fig. 1B) and, with
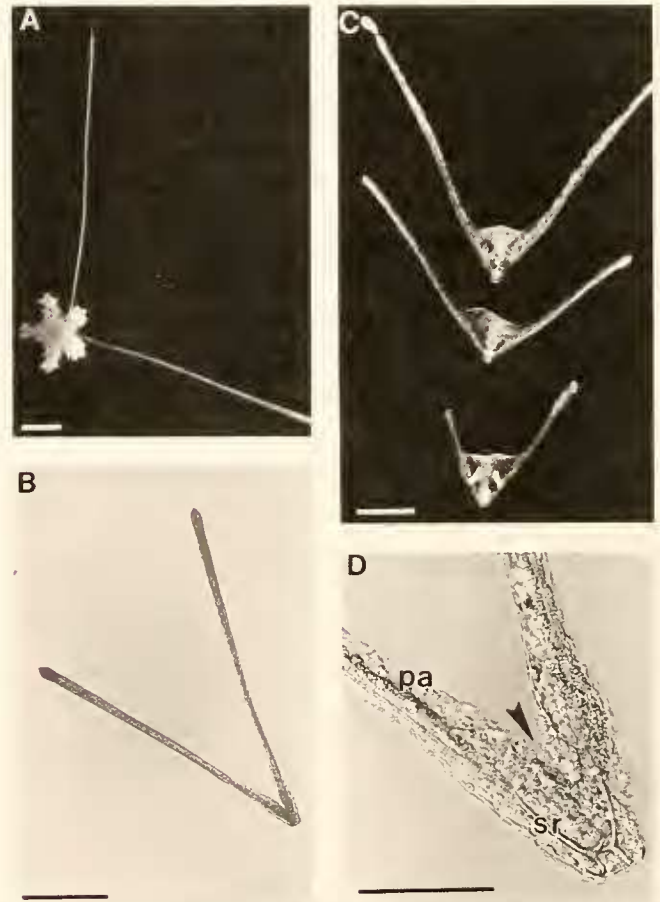

Figure 1. (A) Light photomicrograph of a juvenile *Ophiopholis aculeata* suspended between the outer posterolateral arms. (B) Recently released posterolateral arms from a settled juvenile. (C) A developmental series of three different asexually produced secondary larvae arranged chronologically from top to bottom. (D) Gastrulation of released posterolateral arms. After settlement, the juvenile releases the posterolateral arms (B) which swim using the ciliated epidermis covering the arms (originally part of the primary larval ciliated band). The posterolateral arms (pa) are supported internally by skeletal rods (sr), seen in D. An event similar to gastrulation begins with the invagination of the epidermis (arrowhead) in the vee of the arms. Formation of the secondary pluteus (shown in series in C), is characterized by a shortening of the outer arms and a concentration of tissue between the arms. Scale bars = 0.1 mm.

ciliary band intact, swim off the bottom of the culture dish and into the water column.

The free arms consist of the skeletal rods, mesenchymal cells, a ciliated epidermis, and the blastocoel of the primary larva's posterolateral arms. Within 24 h of release, the tissue forming the inner vee of the arms undergoes a process similar to gastrulation. This results in the production of an archenteron that continues to develop, forming a new larval gut (Figs. 1C, D and 2A). As in development of the primary larva, the archenteron gives rise to an anterior coelom that divides and grows to produce the right and left axohydrocoels (Fig. 2B). The formation of the somatocoels in both primary and secondary larvae is, at present, unclear. Over the course of 3 to 4 days, the posterolateral arms regenerate the structures typical of an ophiopluteus, including the gut, the coeloms, and additional feeding arms (Figs. 1C, 2C).

Prior to feeding in the secondary larva, tissue and energy resources appear to be supplied by the posterolateral arms, as indicated by a decrease in the length of the arms
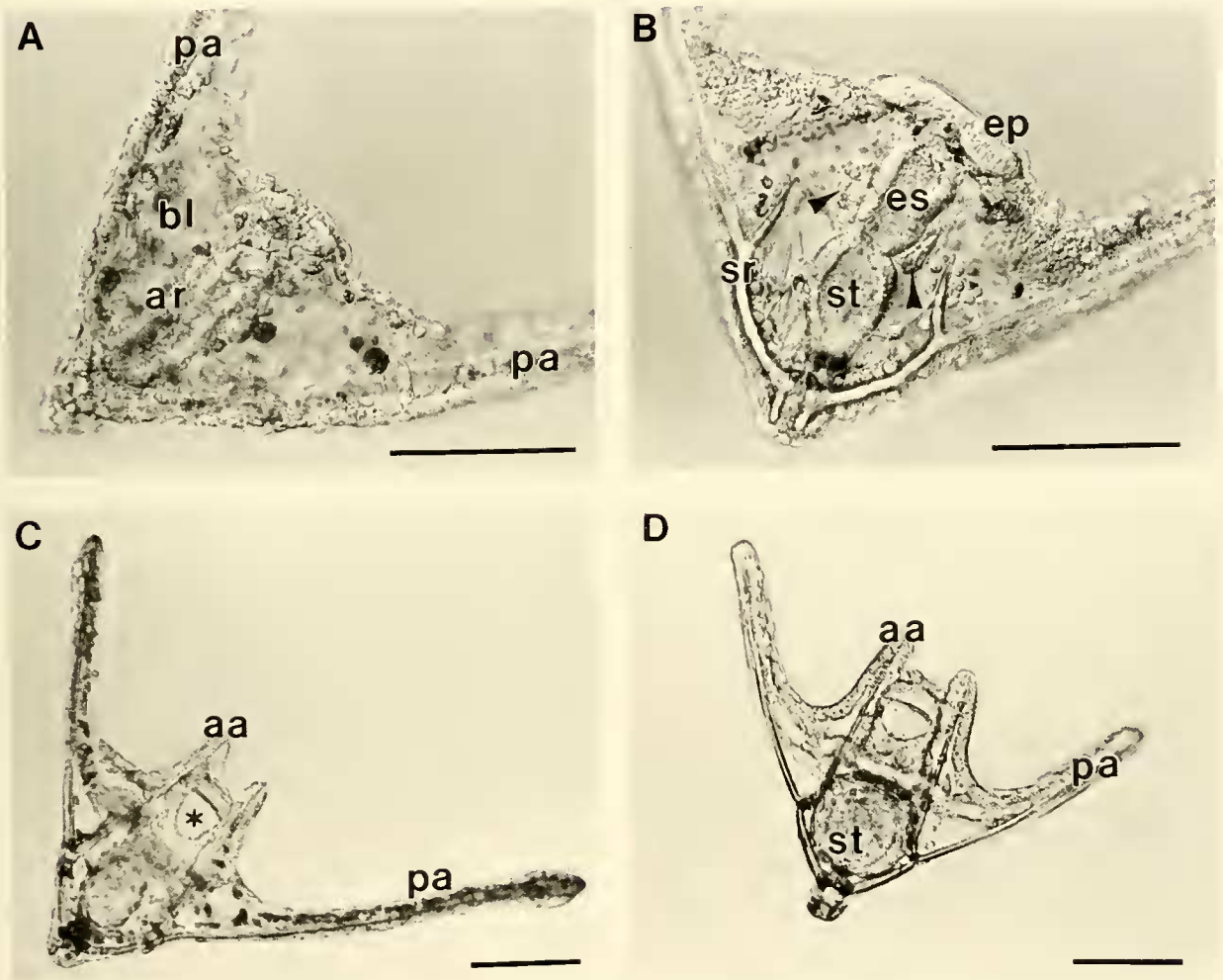

Figure 2. (A, B) Light photomicrographs of the formation of the larval gut in 2-arm clones of *Ophiopholis aculeata.* (C) Photomicrograph of a 4-arm pluteus produced from released posterolateral arms of primary juvenile. (D) Photomicrograph of a primary pluteus resulting from fertilization of an ovum and development of the zygote. In cloned larvae, the archenteron (ar) gives rise to the esophagus (es), stomach (st), and intestine (not shown). The mouth (asterisk) opens from the exterior into the esophagus, but it is not known if it forms at the opening of the archenteron (blastopore analog) or elsewhere. As in primary larvae, the anterior coelom arises from the tip of the archenteron and later divides to form the right and left axohydrocoels (arrowheads). The 4-arm asexually produced pluteus (C) is morphologically similar to a primary pluteus (D) except for increased pigment in the epidermis (seen as dark spots in this micrograph) and disproportionately longer posterolateral arms (pa). aa, anterolateral arm; bl, blastocoel; ep, epidermis; sr, skeletal rod. Scale bars = 0.1 mm.

(Fig. 1C). The average total length (sum of left and right arm lengths) for arms just released from the juvenile was 620 ± 71 μm (mean $\pm$ 1 SD, $n = 6$); in contrast, the mean total arm length of 2-arm prefeeding secondary larvae was 374 ± 82 μm ($n = 6$). This 40% difference represents a significant decrease ($t = 5.53$, $P < 0.001$) in the average total arm length. During reformation of the secondary pluteus, the epidermis covering the arms appears to be pulled towards the inner vee of the arms, presumably providing the cells for invagination and formation of the archenteron. The epidermis appears buckled along the length of the arms and is pulled away from the ends of the skeletal rods. In some larvae, the reduction in arm length was not symmetrical, with one arm being shorter than the other (Fig. 1C). The reason for this asymmetry is not known, but it apparently has no adverse effect on development. These larvae formed normal plutei and the asymmetry disappeared as development progressed.

Within 5 days, a feeding 4-arm pluteus is formed (Fig. 2C). This larval clone is morphologically indistinct from a primary larva except for having more of a characteristic orange pigment in its epidermis (seen in fully developed primary larvae) and disproportionately long posterolateral arms (compare Fig. 2C with 2D). Continued growth and morphogenesis results in an 8-arm pluteus that is indistinguishable from the primary larva at the same developmental stage. Metamorphosis of the secondary larva begins with the formation of the hydrocoelic lobes and follows a morphogenic pattern similar to that of the primary larva. At the time of settlement, the secondary juvenile retains the posterolateral arms but, as in the primary larva, eventually releases them. The free arms return to the water column and begin a tertiary cycle of development that results in another planktonic ophiopluteus.

Ophioplutei collected from plankton tows

Plankton tows taken off the coast of Ft. Pierce, Florida, contained a series of developmental stages of an unknown ophiuroid larva. These stages included 8-arm plutei (Fig. 3A), metamorphosed juveniles suspended between the posterolateral arms, free-swimming posterolateral arms (Fig. 3B), and various stages of regenerating posterolateral arms (Fig. 3C, D). Juveniles maintained in culture dishes eventually settled and released the posterolateral arms which, as in *O. aculeata*, began morphogenetic changes consistent with regeneration of the larva.

Stages collected from the plankton showed a progressive series from recently released arms (Fig. 3B) to an early 4-arm pluteus (Fig. 3D). In some, but not all, of the recently released stages the two arms were of different lengths (Fig. 3B). This asymmetry had no noticeable adverse effect on swimming or development and disap-

peared by the 4-arm stage. In plankton-collected clones, no asymmetry in arm length was observed in 4-arm secondary larvae.

Gastrulation by posterolateral arms of this east coast species may not occur as it does in *O. aculeata*. At the junction of the two posterolateral arms, the transverse rods form a spherical cage (Fig. 3C). Although not clearly seen in Figure 3C, the lumen of this cage is filled with cells. It is not known from where these cells originate or if they reform the larval gut, and subsequently the larval coeloms. Asexually produced 4-arm plutei appeared to have disproportionately longer posterolateral arms than those expected for primary plutei at a similar developmental stage, but without direct comparison, this remains a subjective observation. Beyond the 4-arm stage, secondary plutei were indistinguishable from primary larvae.

Discussion

In *O. aculeata*, tissues in the asexually produced larvae appear to originate from dedifferentiation and redifferentiation of the epidermis covering the posterolateral arms. Mesenchymal cells are present in the blastocoel (of the arms), but are associated with the skeletal rods and do not seem to contribute to the formation of the archenteron or the axohydrocoels. This developmental sequence is similar to that seen in the blastula of *O. aculeata* and raises questions concerning the ontogeny of larval morphogenesis. Principally, do the clones follow the same genetic and morphogenetic pathways as the primary larva or do they use a novel developmental pattern?

Specific questions about the development of asexually produced larvae are how the larval body axes are established in the clone and what mechanisms are involved in differentiation of endoderm and subsequently mesoderm from the ectodermal epithelium covering the free-swimming arms. In both primary and secondary larvae, the archenteron forms along the long axis of the larval skeleton (anterior-posterior axis), but it is not yet known if the mouth in the secondary larva forms at the site of invagination or at a point 90 degrees from the advancing tip of the archenteron, as in the primary larva. If, after invagination of the arm epithelium, the cells forming the archenteron in the cloned pluteus are embryologically equivalent to those of the primary gastrula, formation of endodermal and mesodermal structures could simply repeat the developmental program of the primary embryo.

O. aculeata and the ophiuroid species collected from the plankton may not follow the same developmental sequence in forming a secondary larva—specifically with respect to gastrulation. Although multiple modes of asexual reproduction are reported for asteroid larvae (Jaeckle, 1994), gastrulation appears to proceed from an invagi-
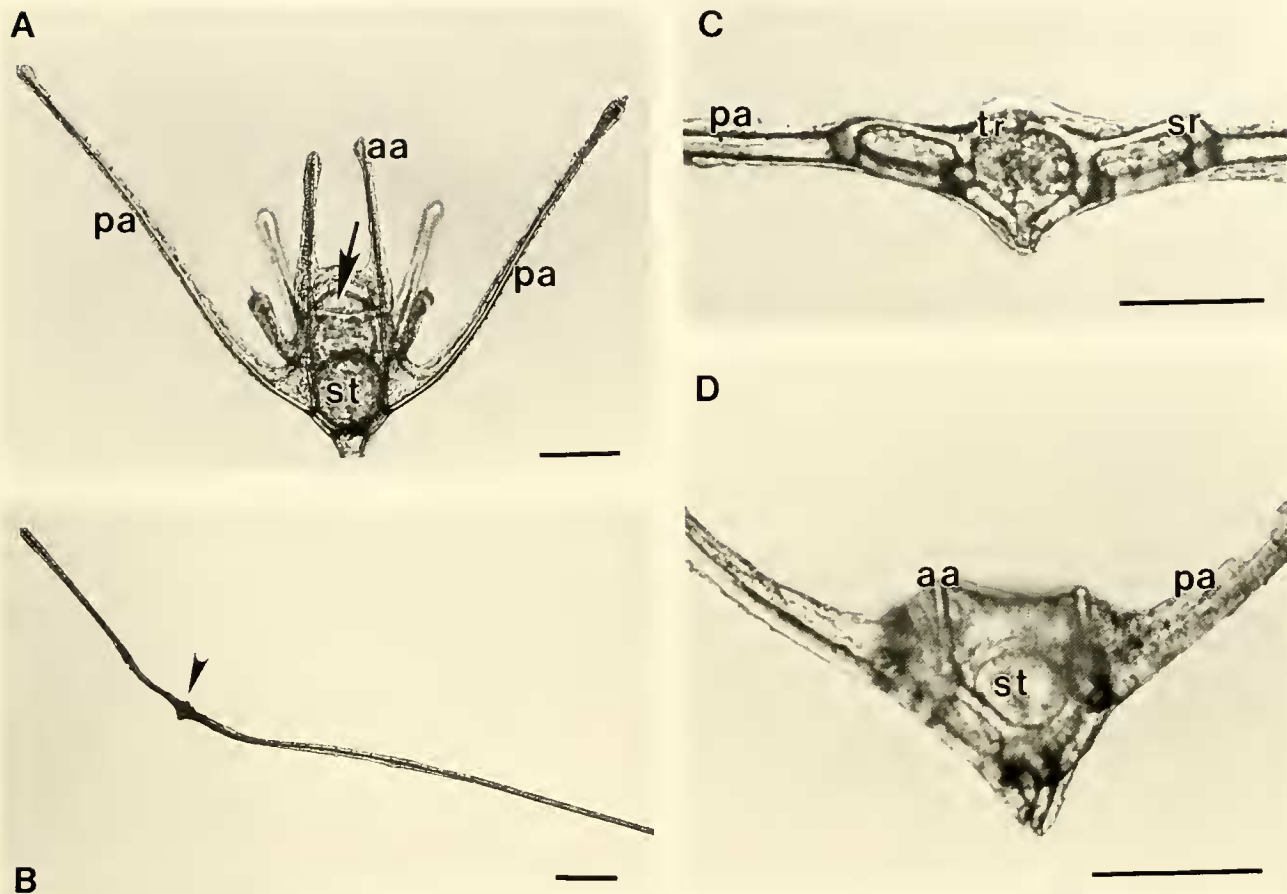
Figure 3. Light micrographs of an ophiopluteus (A), released posterolateral arms (B), and apparently regenerating larvae (C, D) collected from plankton samples taken off the east coast of Florida. Similarities in the larval skeleton and in pigmentation suggest that these specimens represent one species. aa, anterolateral arm; pa, posterolateral arm; st, stomach; sr, skeletal rod; tr, transverse rod; arrowhead, concentration of tissue at the point of contact of the two posterolateral arms. Scale bars = 0.1 mm.

nation of the outer (epidermal) epithelium of the propagule (Bosch *et al.*, 1989; Jaeckle, 1994). The origin of the cells in the vee of the released arms of this unidentified secondary ophiopluteus is at present unknown. They may have originated from invagination of the epidermis of the arms or, alternatively, from mesenchymal cells of the primary larva. If the latter hypothesis is true, the development of the secondary larva deviates from that described for asteroids and for *O. aculeata.*

In ophiuroids, asexually produced larvae can complete metamorphosis and initiate another generation of planktonic larvae. The formation of tertiary larvae suggests that the production of asexual larval clones could continue indefinitely as long as environmental conditions could support a planktonic existence. Development of secondary larvae is known for *Ophiopluteus opulentus* (Mortensen, 1921) and *Ophiopholis aculeata* (this manuscript). Mladenov (1979) reported the release of the posterolateral

arms by the settled juveniles of *Ophiothrix oerstedi.* In this species, however, the arms did not develop and eventually died. The unidentified pluteus collected off the coast of Florida may be the same larva described by Mortensen (1921) from the West Indies and designated as *O. opulentus* species c. Similarities in the larval skeleton of both larvae and the confluence between the West Indies and the Florida coastline provided by the flow of the Florida Current of the Gulf Stream system raise the possibility that the unidentified larva is *O. opulentus.* Nevertheless, the potential for larval cloning is evident in possibly three species of planktotrophic ophioplutei (although *O. opulentus* is known only from larval stages), and may represent an important life history strategy for planktonic ophiuroid larvae.

The impact of asexual reproduction by larvae on the life history of a species is dependent on the cloned individuals' ability to produce viable gametes, which, in turn,

requires the formation of germ cells. The origin of the primary germ cells in ophiuroids, and in general in echinoderms, is equivocal. Germ cells in echinoderms are thought to arise either from secondary mesenchymal cells or from a proliferation or outgrowth of cells from the epithelial lining of the somatocoel (Gemmill, 1914; Nieuwkoop and Sutasurya, 1981; Delavault, 1966; Houk and Hinegardner, 1980, 1981; Hendler, 1991; Holland, 1991). In ophiuroids, as in other echinoderms, the primary germ cells are not evident in the larval body until late in development, usually just prior to or after metamorphosis (Gemmill, 1914; Houk and Hinegardner, 1980, 1981; Smiley, 1986; Hendler, 1991; Holland, 1991). The ''absence'' of germ cells in earlier developmental stages suggests, concurrently or alternatively, a delay in the induction of the expression of germ cell characteristics and an origin of the primary germ cells from structures not formed until later in development.

The formation of primary germ cells in asexually reproduced larvae has not been demonstrated, but the following three hypotheses are possible. The cloned larvae lack the ability to produce germ cells and, therefore, form sterile adults. Primary germ cells are sequestered from a population of stem cells in the primary larva and are transferred to the secondary (or subsequent) propagule prior to its release from the primary larva. Finally, the primary germ cells could arise *de novo* from the somatocoelic epithelium, which apparently arises anew in the cloned larva. The latter two hypotheses are consistent with the formation of the germ cells in primary larvae either from mesenchyme or from the epithelium of the somatocoel.

Provided that the juveniles resulting from cloned larvae can produce viable gametes, asexual reproduction of larval propagules increases the effective population size without additional reproductive cost to the primary adult. Further, asexual reproduction by larvae may produce a larger increase in the number of juveniles than could be realized from an equivalent increase in the number of eggs released by the female. The loss of individuals during larval development (mortality rate > 0) requires that the number of eggs released by the female exceed the number of juveniles produced.

Cloned larvae, which enter the water column at a size equivalent to the arm-span of an 8-arm pluteus, may experience a decrease in predation (Rumrill *et al.*, 1985; Pennington *et al.*, 1986; Rumrill, 1987), and thus a lower mortality rate, compared to less developed stages. The reduction may, however, depend on the type of predator encountered. In the presence of arthropod and chaetognath predators, blastulae and prism stages of *O. aculeata* were more susceptible than 4-arm plutei, which were more susceptible than 8-arm plutei (Rumrill, 1987). In contrast, when larvae were presented to a copepod or hydromedusa predator, a greater percentage of later larval stages was lost to predation (Rumrill, 1987). Nevertheless, decreased predation resulting from morphological or behavioral characteristics of more advanced larval stages (Pennington *et al.*, 1986) may in some cases improve the survivorship of asexually produced plutei.

For each juvenile that survives to settlement, an additional larva is produced; this not only increases the number of individuals of a given genetic lineage, but also enhances the dispersal potential of the species. Subsequent generations of asexually produced larvae have the potential to increase the distance of dispersal by at least as much as the primary larva. Dispersal promotes genetic connectivity between populations geographically isolated by distance and allows recruitment to new habitats (Chia, 1974; Crisp, 1974; Strathmann, 1974; Scheltema, 1986). Asexual reproduction by planktonic larvae increases the life span of a genetically identical cohort and distributes the timing of the attainment of competence to settle. If appropriate settlement sites are randomly distributed along a dispersal axis, then asexual propagation—by producing more larvae that can spread over a wider area—increases the likelihood that a competent larva will locate a suitable settlement site.

Acknowledgments

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